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Supplemental Sewage Scum and Organic Municipal Solid Waste Addition to the Anaerobic Digestion of Thickened Waste Activated Sludge: Biomethane Potential and Microbiome Analysis

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Abstract: Sewage scum (SS) is collected from sedimentation tanks in wastewater treatment plants (WWTPs). Despite its huge biogas potential, there is limited information on its potential as a co-substrate and microbial ecology, especially during anaerobic co-digestion (ACo-D) of the organic fraction of municipal solid waste (OFMSW) and thickened waste activated sludge (TWAS). In this biomethane potential (BMP) study, the bioenergy yield achieved by the supplemental addition of SS and OFMSW to TWAS was investigated, along with the microbial ecology. Compared with the digestion of TWAS alone, which produced 184.6 mLCH4 gVS^{-1}, biomethane yield was enhanced by as much as 32.4–121.6% in trinary mixtures with SS and OFMSW, mainly due to the positive synergistic effect. Furthermore, a mixture of 40%SS + 10%TWAS + 50%OFMSW produced the highest biogas yield of 407 $mLCH_4$ gVS^{-1}, which is proof that existing WWTPs can produce additional energy by incorporating external bioresources, thereby reducing greenhouse gas emissions. Modified Gompertz and logistic function estimates showed that methane production rate improved by as much as 60% in a trinary mixture compared with the digestion of TWAS alone. The genus *Methanosaeta*, capable of generating methane by the acetoclastic methanogenic pathway among all the archaeal communities, was the most prominent, followed by hydrogenotrophic methanogen *Methanospirillum*.

Keywords: biomethane potential; co-digestion; microbiome analysis; organic fraction of municipal solid waste (OFMSW); sewage scum; thickened waste activated sludge (TWAS)

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1. Introduction

Climate change, environmental challenges, and the need to reduce carbon footprints and greenhouse gas (GHG) emissions have all boosted the global demand for renewable energy by at least a factor of two to three over the last couple of decades [1]. Renewable energy generation in the form of biogas production through anaerobic digestion (AD) of organic waste can limit GHG emissions and potentially turn wastewater treatment plants (WWTP) into net energy producers rather than energy consumers. However, there are lingering challenges of huge sludge production volume, poor biogas generation rate, low energy content, increased energy consumption, and increased biogas upgrading costs. On top of that, the design capacity for most digesters is often not met until the end of the design life of the plant (approximately 20–25 years) [2]. This scenario has forced many WWTPs to operate oversized digesters underloaded by 15 to 30% [3], especially at the early stages of operation, thereby missing a great opportunity to maximize energy production. Current and future realities, however, require a search for supplemental energetic feedstocks to boost biogas quality and quantity in order to fully utilize digester capacity [4].

WWTP sludge is composed of primary sludge and secondary sludge (waste activated sludge, WAS), which is typically rich in micro/macronutrients and alkalinity [4–6]. However, the recovery of biogas from WAS is negatively impacted by its low C/N ratio, low

biodegradable organic matter content, and difficulty during digestion. Specifically, ACo-D with WAS has been reported as a promising strategy to resolve some of these challenges to an extent. At the same time, it can improve biogas production and enhance the efficiency of organic waste degradation and the energy efficiency in a WWTP [6,7]. Moreover, ACo-D can be implemented without a major capital investment because existing infrastructures can be utilized [8].

The co-digestion of WWTP sludge with an organic fraction of municipal solid waste (OFMSW) appears to be the most reported in the literature. OFMSW is characterized by high solid concentration and high C/N ratio, which can balance nutrients, dilute toxicants, improve kinetics, and enhance the microbial community [6,9–14]. Results suggest that the totality of these benefits has led to process improvements and enhanced biomethane recovery when treated with WWTP sludge [9–11]. However, the ACo-D of WWTP sludge with other wastes such as fruit and vegetable waste [15,16]; manure [13,17]; landfill leachate [18]; and various fat, oil, and grease (FOG)/lipid-rich wastes [8,19–21] have also been investigated as sources of external organics in a WWTP. From an energy standpoint, FOG wastes are one of the most attractive co-substrates because of their high theoretical methane potential (up to 1 m^3 CH_4 $kgVS^{-1}$) [19–22]. Nonetheless, such wastes in high concentration can cause process instability and inhibit methanogenesis due to the accumulation of long-chain fatty acids (LCFA) [19–22]. FOG wastes are also known to vary differently in composition and chemical characteristics depending on the source, mechanism of collection, abatement device configuration, the device pumping frequency, etc. [22]. These factors may affect biogas quality and quantity during AD and ACo-D.

One of the factors required for synergism during ACo-D is the proper mixing ratio of the substrate and the co-substrate. The use of FOG wastes as tertiary external organics in the proper mixing ratio has been previously explored as an alternative strategy for enhancing biogas production from WWTP sludge [19,23]. For instance, the co-digestion of sewage sludge, grease trap sludge (GTS) and OFMSW at a volatile solids (VS) ratio of 40%:30%:30%, respectively, improved methane production by as much as 130% over treatment of sewage sludge alone [19]. Ferreira et al. [24] observed the co-digestion of sewage sludge, food waste (FW), and residual glycerol combined in the ratio 89.6:10.0:0.4 (v/v%) in a pilot semi-continuous digester led to a 1.8-fold increase in methane yield when compared to the digestion of sewage sludge alone. Finally, methane production from sewage sludge was enhanced by 3.8-fold through the addition of 3% (v/v) crude glycerol and food waste [23]. So far, the use of sewage scum (SS) as a supplemental source of FOG and energy during co-digestion of OFMSW and sewage sludge is yet to receive enough attention in the literature. SS is collected from the surface of primary settling tanks, secondary treatment, and secondary settling tanks in WWTPs, and the common method of disposal is landfilling due to inefficient recycling and treatment methods [25].

Considering the above, further investigations focusing on the feasibility and optimizing biomethane recovery during supplemental addition of SS and OFMSW to underloaded WWTP sludge digesters is of interest and can potentially serve as a viable alternative to generate renewable energy and decarbonize WWTPs. One common way to assess this is to perform biomethane potential (BMP) tests on the feedstocks. Furthermore, the characterization of the microbial community during trinary co-digestion could unravel the microbial dynamics, microbial community stability, and mechanism behind methane production, especially during changes in substrate proportions in a mixture. The influence of substrate proportion on the microbial community during the addition of SS and OFMSW to sewage sludge will also provide new information. Accordingly, this study was carried out to (i) investigate the feasibility of the supplemental addition of SS and OFMSW through co-digestion with thickened waste activated sludge (TWAS) in a WWTP scenario, (ii) assess the overall process performance in terms of biomethane production and synergistic/antagonistic effect, (iii) apply relevant kinetic models to interpret the experimental results, and finally (iv) investigate the impact of the different substrate proportions on the microbial community.

2. Materials and Methods

2.1. Materials

The sewage scum (SS) used in this study was collected from the surface of a primary sedimentation tank of the WWTP in Gatineau, QC, Canada. The thickened waste activated sludge (TWAS) was collected from the same plant. The inoculum used was collected from a mesophilic digester of Robert O. Pickard Environmental Center, Ottawa, ON, Canada. The inoculum was degassed at 40 ± 2 °C for one week before BMP preparation to remove endogenous biomethane [26]. OFMSW was collected from the compost facility of a landfill site in Moose Creek, ON, Canada. The OFMSW was mixed with tap water and homogenized using an electric blender. No other treatment was carried out on any of the samples. All collected wastes were transported safely into the laboratory and stored at a temperature of 4 °C until they were used.

2.2. Experimental Design

Table 1 shows the experimental design used in this study. In the first set, single substrate BMP tests were conducted to determine the methane potential of all individual substrates; SS, OFMSW, TWAS, and inoculum were set as control. For the second set, trinary co-digestion experiments based on VS were conducted with 20% SS additions to TWAS and OFMSW in four ratios (10:70, 30:50, 50:30, 70:30). Lastly, another set of trinary co-digestions experiments with 40% SS (VS basis) additions with TWAS and OFMSW present in three VS ratios (10:50, 30:30, and 50:10) were also carried out. The lower SS% was selected due to feasibility considerations regarding the low production of SS.

Table 1. The experimental design used in this study.

Sample [1]	Inoculum (mL)	SS (g)	TWAS (mL)	OFMSW (g)	ISR [2]	Initial VS (g)
		Mono-Digestion				
SS	205	3.5	0.0	0.0	2	4.83
TWAS	205	0.0	62.6	0.0	2	4.83
OFMSW	205	0.0	0.0	38.1	2	4.83
Inoculum	205	0.0	0.0	0.0	-	3.22
		Trinary Co-Digestion				
20%SS + 10%TWAS + 70%OFMSW	205	0.7	6.3	26.7	2	4.83
20%SS + 30%TWAS + 50%OFMSW	205	0.7	18.8	19.1	2	4.83
20%SS + 50%TWAS + 30%OFMSW	205	0.7	31.3	11.4	2	4.83
20%SS + 70%TWAS + 10%OFMSW	205	0.7	43.8	3.8	2	4.83
40%SS + 10%TWAS + 50%OFMSW	205	1.4	6.3	19.1	2	4.83
40%SS + 30%TWAS + 30%OFMSW	205	1.4	18.8	11.4	2	4.83
40%SS + 50%TWAS + 10%OFMSW	205	1.4	31.3	3.8	2	4.83

[1] % is based on TVS; [2] Inoculum to substrate ratio based on TVS.

All BMP experiments were conducted in a 500 mL glass serum bottle with a working volume of 350 mL. A headspace of 30% was maintained in all bottles to prevent CO_2 absorption. All BMP bottles were prepared in duplicates to ensure reproducibility, and each bottle contained a total of 4.83 g VS load with an inoculum-to-substrate ratio (ISR) of 2 (gVS/gVS). Inoculum without the addition of any substrate was set as the control. No supplemental nutrients or alkalinity were added to the bottles because the inoculum is a viable source of micronutrients, trace elements, and vitamins, as well as a pH buffer for the biodegradation of substrates. The bottles were purged with nitrogen gas for about two minutes to maintain anaerobic conditions and expel oxygen from the headspace. The bottles were then sealed using self-healing caps and silicon. The bottles were agitated on a New Brunswick Scientific Controlled Environment Incubator Shaker Model G-25 rotating at 110 rpm at 40 ± 2 °C. The test was terminated after day 43, when the daily biogas production for three consecutive days was observed to be less than 1% of the cumulative biogas production.

2.3. Analytical Procedure

Biogas production from the BMP bottles was measured daily using a U-tube manometer and converted to standard temperature and pressure (STP) (0 °C, 1 atm). The net biogas volume from all the BMP bottles was obtained by deducting the biogas contribution of the inoculum (control). Weekly biogas composition was conducted using a gas chromatograph (Series 400, Gow-Mac Instrument Co., Bethlehem, PA, USA) fitted with a HayeSep® N 80/100 mesh, (5′ × 125′) and Molesieve equipped with a thermal conductivity detector (TCD). The HayeSep column N80/100 was used for N_2, CH_4, and CO_2 percentage analysis, using helium as the carrier gas. The temperatures of the column, detector, and injector were maintained at 35 °C, 185 °C, and 50 °C, respectively, during runs. pH was determined using a HQ40d portable multi-parameter meter fitted with an Intellical PHC201 gel-filled pH electrode (HACH, Loveland, CO, USA). Total solids (TS) and volatile solids (VS) of the substrates were determined according to standard method 2540 G [27]. Alkalinity determination was based on TNTplus™870 reagent vials (Method 10239, HACH, Loveland, CO, USA). COD was conducted according to TNTplus™ 823 (Method 10212, HACH, Loveland, CO, USA). TAN was determined based on TNT plus™ 832 reagent vials (Method 10205, HACH, Loveland, CO, USA), while VFA was determined based on TNT plus™ 872 reagent vials (Method 10240, HACH, Loveland, CO, USA).

DNA Extraction, Sequencing, and Sequence Processing

Selected reactor samples were immediately collected on day 1 (initial) and day 43 (final) of the experiment for shotgun metagenomic sequencing. Metagenomic DNA was extracted using a Qiagen MagAttract PowerSoil DNA KF kit with a KingFisher robot. The quality and integrity of the DNA were visually evaluated through agarose gel electrophoresis and quantified with a Qubit 3.0 fluorometer (Thermo-Fischer, Waltham, MA, USA). Genomic libraries were prepared with an Illumina Nextera library preparation kit (Illumina, San Diego, CA, USA). The libraries obtained were paired-end sequenced (2 × 150 bp) using NextSeq 500 in medium-output mode (Illumina, San Diego, CA, USA). Shotgun metagenomic sequence reads were processed per standardized protocols. Reads in FASTQ format were quality-filtered (leading: 3, trailing: 3, sliding window: 4:15, minlen: 36) and adaptors were removed using Cutadapt v2.6 [28], while trimming was performed with Trimmomatic software (v0.36) [29]. Low-complexity sequences were detected with Komplexity v0.3.6 [30].

2.4. Performance Indicators

The performance of both single substrate and co-digestion were assessed using the following indicators in various sections:

2.4.1. Volatile Solids Removal (%VSR)

The %VSR was calculated using Equation (1)

$$\%VSR = (VS_{initial} - VS_{final}) \times 100/VS_{initial} \quad (1)$$

where $VS_{initial}$ and VS_{final} are the VS measured before and after the digestion process.

2.4.2. Synergistic Effects

While BMP assays do give experimental results from a co-digestion study, synergistic effects do occur due to the inner reactions of the various components in a co-digestion study. To evaluate possible synergistic effects produced during co-digestion as well as to understand the influence of each substrate in the various mixtures, equation 2 was used [31].

$$\alpha = \text{Net Experimental CMY}/CY_{calculated} \quad (2)$$

where α is the synergistic index. If $\alpha > 1$, the co-digestion mixture has a synergistic effect in the net experimental CMY; if $\alpha = 1$, the substrates work independently from the co-digestion

mixture; if $\alpha < 1$, the co-digestion mixture has a competitive or antagonistic effect in the final mixture [31]. The net experimental CMY is the cumulative methane yield from the BMP tests from each co-digestion mixture (mL gVS^{-1}), while the $CY_{calculated}$ is estimated from the BMP of the sole substrates considering the VS of each substrate contained in the final mixture (mL gVS^{-1}).

2.4.3. Kinetics model Application

Three commonly used kinetic AD models were applied and assessed in this study; first-order equation (FO), modified Gompertz model (GM), and logistic function (LF), shown in Equations (3)–(5), respectively [32].

$$\beta(t) = \beta_o\ [(1 - e^{-kt})] \quad (3)$$

$$\beta(t) = \beta_o \exp\ (-\exp\ ((R_m\ e)/\beta_o\ (\lambda - t) + 1)) \quad (4)$$

$$\beta(t) = \beta_o/(\{1 + \exp\ [(4R_m)/\beta_o(\lambda - t) + 2]\}) \quad (5)$$

where $\beta(t)$ is the cumulative biogas production (mL gVS^{-1}) at a specific time, t is the time (hour or day), β_o is the ultimate production potential (mL gVS^{-1}), R_m is the methane production rate (mL gVS^{-1} d^{-1}) or (mL gVS^{-1} hr^{-1}), λ is the lag phase (hour or day), and e = 2.7183. k is the hydrolysis rate constant (hr^{-1}) or (d^{-1}) [8]. λ, β_o, R_m, and k were fitted using the generalized reduced gradient non-linear regression algorithm of solver in Microsoft Excel with minimum residual sum of squared errors between experimental data and the model curve [33]. Maximum number of iterations was set at 100 in all cases.

3. Results

3.1. Substrate Characteristics

Table 2 shows the various characteristics of the substrates and inoculum used in this study. SS is seen to have a higher VS content compared to TWAS and OFMSW, an evidence of its huge biogas potential. However, the VS of TWAS is comparable with 2.11–2.21% observed by [19]. The pH values of the feedstocks are all within the acidic scale, which may be related to the higher VFA concentrations. Lower VFA concentration and a near-neutral pH in the inoculum are indicative of its methanogenic state.

Table 2. Characteristics of the substrates and inoculum.

Parameter	TWAS	OFMSW	Sewage Scum	Inoculum
COD (mg L^{-1})	66,950 ± 450	42,400 ± 100	28,965 ± 935	21,600 ± 100
pH	5.7 ± 0.02	5.0 ± 0.0	4.8 ± 0.03	7.7 ± 0.02
TAN (mg L^{-1})	215 ± 3	74 ± 2.65	93.8 ± 0.4	1320 ± 3
VFA (mg L^{-1})	5670 ± 30	16,100 ± 201	11,835 ± 45	257 ± 10
Alkalinity (mg $CaCO_3$ L^{-1})	4475 ± 55	3195 ± 55	1515 ± 95	7330 ± 42
TS (g kg^{-1})	33.2 ± 0.19	51.65 ± 4.82	462.3 ± 19.5	23.17 ± 0.74
VS (g kg^{-1})	25.66 ± 0.15	42.17 ± 4.59	453.5 ± 19.9	15.7 ± 0.02
VS/TS	0.77 ± 0.01	0.82 ± 0.02	0.98 ± 0.00	0.68 ± 0.01

± represents standard deviation based on three samples.

3.2. Biomethane Production

3.2.1. Mono-Substrate Digestion

Figure 1 shows the average net cumulative biomethane yield (CMY) from the mono-substrate digestion of the various substrates. A 5% standard error was applied to all plots. During AD, methane production occurs at the methanogenesis stage. Net CMY (and average methane concentration) of 625.4 mL gVS^{-1} (61.5% CH_4), 252.3 mL gVS^{-1} (51.8% CH_4), and 184.6 mL gVS^{-1} (54.4% CH_4) were produced from SS, OFMSW, and TWAS, respectively. The results show that SS had the highest biomethane yield, and thus suited as a co-substrate during ACo-D. The high biomethane yield of FOG wastes has been

associated with the high number of carbon and hydrogen atoms as well as the C:N ratio, which can be as high as 22 [22]. However, the hydrolysis of FOG wastes forms LCFA, which is known to compromise methanogenesis when present in high concentration [19–22]. It is important to note that SS was not present in high concentrations in this study. The methane yield of SS in this study is within the range of 430–990 mL gVS^{-1} reported in similar studies involving FOG [5,19,34]. CH_4 yield of 252.3 mL gVS^{-1} from OFMSW falls within the range of 186–570 mL gVS^{-1} that has been reported in some literature [12,35].

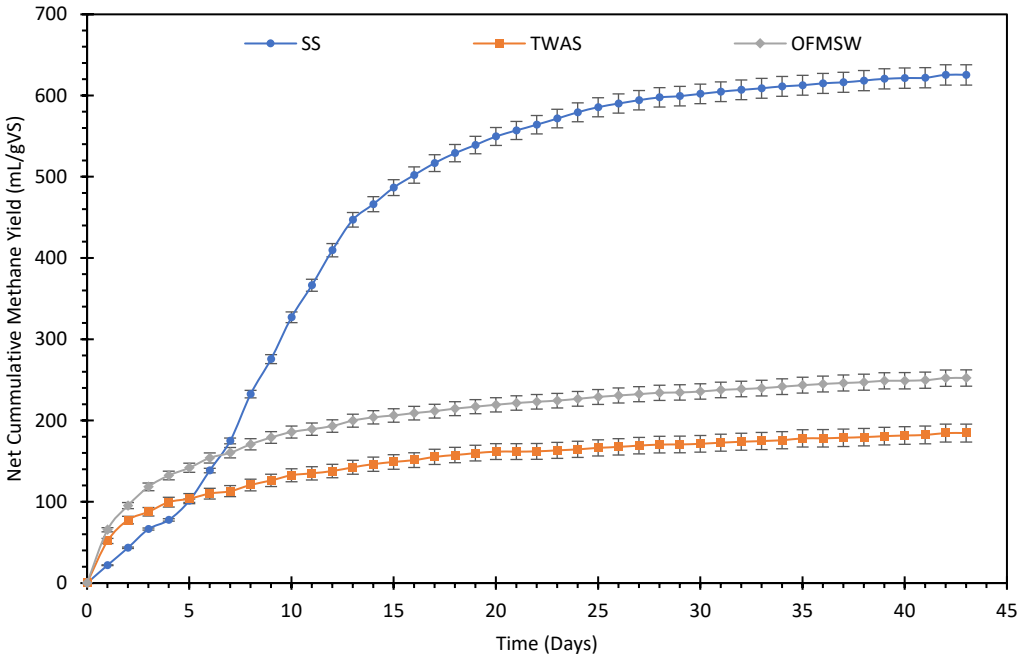

Figure 1. Average net cumulative methane yield (CMY) from the various single substrates used in this study.

The methane yield of TWAS in this study (184.6 mL gVS^{-1}) was the lowest of the three feedstocks and falls within some previously reported values of 143–220 mL gVS^{-1} [36]. Compared with the other substrates in this study, the lower methane yield of TWAS was expected and has been suggested to relate to its low VS content, low organic load, or low C:N ratio, which is usually between 6 and 9. Therefore, the above results indicate that supplementing TWAS with SS and OFMSW can potentially enhance methane production during ACo-D. Despite the differences in methane yield, no significant differences were observed in the %VSR of the feedstocks because the values were within the range of 34.4%–40.4% (Table 3).

3.2.2. Co-Digestion Mixtures

The net CMY from the various co-digestion mixtures involving supplemental addition of SS and OFMSW to TWAS are represented in Figure 2. In Figure 2, 40%SS + 50%TWAS + 10%OFMSW and 40%SS + 30%TWAS + 30%OFMSW experienced a distinct lag time (shown later in Table 4) before biomethane production was substantial. This observation might be related to metabolic adjustments and pattern of preferential consumption of a substrate with a higher surface ratio (TWAS) before a lower surface ratio substrate (SS and OFMSW). The presence of multiple substrates in a digestion medium may trigger a diauxic pattern of microorganism growth. A similar diauxic pattern was observed during the co-digestion of low carbohydrate with high-fat agricultural wastes [37].

Table 3. BMP test results in both mono-digestion and co-digestion experiments.

Substrate	Net CMP (mL)	Net CMY (mL gVS^{-1})	$CY_{calculated}$ (mL/gVS)	% Increase (Net CMY − CY)/(CY) × 100	α	Average $\%CH_4$	VSR (%)
SS	1006.3	625.0	625.0	-	-	61.5	35.3
TWAS	297.2	184.6	184.6	-	-	54.4	34.4
OFMSW	406.3	252.3	252.3	-	-	51.8	40.4
20%SS + 10%TWAS + 70%OFMSW	539.8	335.3	320.1	4.7	1.05	51.3	46.1
20%SS + 30%TWAS + 50%OFMSW	411.5	255.6	306.6	−16.6	0.83	56.9	33.5
20%SS + 50%TWAS + 30%OFMSW	566.1	351.6	293.0	20.0	1.20	56.8	36.9
20%SS + 70%TWAS + 10%OFMSW	393.6	244.5	279.5	−12.5	0.87	56.6	34.9
40%SS + 10%TWAS + 50%OFMSW	657.5	408.4	376.2	8.6	1.09	58.8	35.6
40%SS + 30%TWAS + 30%OFMSW	602.1	374.0	381.1	−1.9	0.98	56.5	29.9
40%SS + 50%TWAS + 10%OFMSW	573.8	356.4	367.5	−3.0	0.97	55.7	29.3

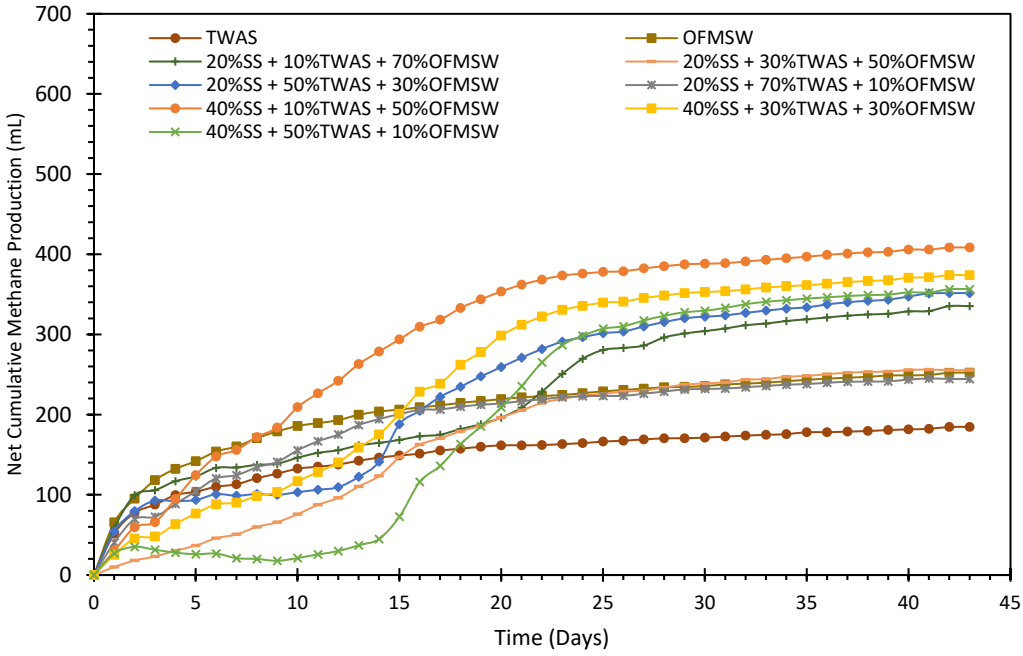

Figure 2. Comparison of the cumulative methane yield from TWAS and OFMSW with trinary co-digestion mixtures.

Biomethane production can be expected to improve when complementary substrates are co-digested due to positive synergism, substrate diversity, dilution of inhibitory components, improved nutrient presence, and higher biodegradable components [2,9–12]. The result of this study shows all ACo-D mixtures produced higher methane yields than TWAS alone. Based on the net CMY from TWAS alone (184.6 mL gVS^{-1}), net methane yield was enhanced by at least 32.4% in 20%SS + 70%TWAS + 10%OFMSW (244.5 mL gVS^{-1}) and as much as 121.4% in 40%SS + 10%TWAS + 50%OFMSW (408.4 mL gVS^{-1})]. The improvement in methane yield shows that it is beneficial to add SS and OFMSW to TWAS in a WWTP scenario.

Table 4. Comparison of the parameters estimated from the various models.

Sample	Measured CH_4	First Order Model				Modified Gompertz Model					Logistic Function				
		β_o	k	R^2	Δ	β_o	R_m	λ	R^2	Δ	β_o	R_m	λ	R^2	Δ
	mL gVS^{-1}	mL gVS^{-1}	hr^{-1}		%	mL gVS^{-1}	mL gVS^{-1} hr^{-1}	hr		%	mL gVS^{-1}	mL gVS^{-1} hr^{-1}	hr		%
Single substrate Experiment															
SS	625.0	689.8	0.00292	0.985	9.4	614.3	1.81	61.6	0.999	1.7	604.0	1.82	71.7	0.997	3.5
TWAS	184.6	174.1	0.0067	0.978	6.0	168.9	0.76	0	0.947	9.3	168.1	0.7	0	0.936	9.8
OFMSW	252.3	239.0	0.0067	0.984	5.6	231.8	1.08	0	0.956	8.9	230.6	1	0	0.945	9.4
Co-digestion Experiment															
20%SS + 10%TWAS + 70%OFMSW	335.3	401.2	0.00167	0.970	16.4	355.6	0.5	0	0.972	5.7	348.2	0.47	0	0.977	3.7
20%SS + 30%TWAS + 50%OFMSW	255.6	270.6	0.0025	0.983	5.5	261.2	0.53	81.5	0.998	2.1	251.7	0.55	102.4	0.999	1.5
20%SS + 50%TWAS + 30%OFMSW	351.6	385.4	0.00208	0.967	8.8	379.0	0.53	0	0.984	7.2	356.2	0.55	19.95	0.988	1.3
20%SS + 70%TWAS + 10%OFMSW	244.5	243.2	0.00433	0.997	0.5	234.5	0.71	0	0.990	4.3	232.4	0.67	0	0.985	5.2
40%SS + 10%TWAS + 50%OFMSW	408.4	438.8	0.00292	0.996	6.9	406.4	0.89	0	0.998	0.5	398.6	0.86	3.68	0.996	2.5
40%SS + 30%TWAS + 30%OFMSW	374.0	496.9	0.00167	0.981	24.7	387.4	0.72	55.4	0.994	3.5	371.9	0.75	81.4	0.997	0.6
40%SS + 50%TWAS + 10%OFMSW	356.4	-	-	-	-	353.3	1.19	292.2	0.998	0.9	347.5	1.14	294.2	0.997	2.6

The enhancement in methane yield could be due to the positive effect of co-digestion and the increased presence of biodegradable substrates. Grosser [19] observed an 80–128% improvement in methane production over TWAS alone when GTS was co-digested with OFMSW and TWAS. Methane yield appears to increase with an increasing proportion of OFMSW, especially in the 40%SS group. This was, however, not the case in the 20%SS group. A higher fraction of OFMSW (50%VS) and SS (40%VS) most likely created optimum conditions, thus balancing the nutrients and the amount of biodegradable organics. Sosnowski et al. [14] reported biogas production improved with an increase in the fraction of OFMSW present when co-digested with TWAS. In contrast, Xie et al. [38] reported a decrease in biomethane potential with an increasing fraction of food waste (FW) in an FW:SS co-digestion mixture involving 110gFW:150g sewage sludge and 150 gFW:150 g sewage sludge.

The methane concentration in this study (Table 3) was higher in 40%SS + 10%TWAS + 50%OFMSW (58.8%CH_4) than in TWAS alone (51.8%CH_4). Likewise, the VSR of 46.1% and 36.6% was obtained in 20%SS + 10%TWAS + 70%OFMSW and 40%SS + 10%TWAS + 50%OFMSW, respectively. These values are greater than the 34.4% VSR observed in TWAS. From this result, it can be concluded that co-digestion can improve VSR and methane concentration in co-digestion mixtures.

It is, however, important to note that although VSR indicates the extent of microbial activity and degradation during AD, improved methane yield may not necessarily accompany an enhanced VSR of organic substrates, as observed in some trinary mixtures. For instance, relative to the other co-digestion groups involving TWAS, the 46.1% VSR in 20%SS + 10%TWAS + 70%OFMSW did not translate to a higher methane yield. This is consistent with the co-digestion of sewage sludge and GTS from a meat processing plant [39].

In this study, mixtures containing above 40%SS were not considered based on the low production of SS and the feasibility consideration. More importantly, the possibility of digester failure caused by LCFA accumulation exists when high FOG content is digested [19–22]. Shakourifar et al. [20] encountered digester failure beyond 40%VS content of mixed grease trap waste co-digestion with mixed primary sludge and TWAS.

3.2.3. Synergistic Effect during Anaerobic Co-Digestion

Considering the digestion of TWAS alone, the results indicate that CH_4 yield can be enhanced through synergism through a few carefully selected co-digestion scenarios. From Table 3, results show that the synergistic effect was highest in 20%SS + 50%TWAS + 30%OFMSW ($\alpha = 1.2$) for 20%SS addition, and 40%SS + 10%TWAS + 50%OFMSW ($\alpha = 1.09$) for 40%SS inclusion in the co-digestion mixture. When compared to the expected methane yield ($CY_{calculated}$), the synergistic effect translated to an increase of 20% and 8.6%, respectively. These results prove synergism can lead to methane improvement during co-digestion. On the other hand, while the evidence of antagonism was observed in 20%SS + 30%TWAS + 50%OFMSW and 20%SS + 70%TWAS + 10%OFMSW, it was negligible in the case of 40%SS co-digestion mixtures. Going by the $CY_{calculated}$, the trend was to expect an increase in experimental methane yield with an increase in the OFMSW and SS content in the trinary mixtures [19,38]; however, this was not the case. Generally, the α values assumed a similar trend with the net CMY. Overall, the results underscore the importance of conducting experimental work to determine the actual methane yield of substrates and co-substrates rather than relying on theoretical methods. The use of BMP during ACo-D could serve as a screening tool useful for co-substrate evaluation.

3.2.4. Total VFA (TVFA) to TA Ratio, pH, and TAN

The average total VFA/TA in both mono and co-digestion modes is shown in Figure S1A in the Supplementary Information (SI). Feng et al. [40] identified three phases in assessing AD; stable (<0.4), some instability (0.4–0.8), and significant instability (>0.8). However, no universal value for total VFA/TA or consensus exists because AD systems vary significantly, even when the same substrates are treated [41]. Even with the same

parameter, multi-thresholds exist [41]. Figure S1A shows that OFMSW as a single substrate experienced some instability at the initial stage with a VFA/TA ratio of 0.5 and at the final stage when the VFA/TA ratio of 0.4 was recorded. Similarly, the VFA/TA ratio was initially between 0.4 and 0.6 in the 40SS% mixtures but returned to acceptable levels (<0.3) at the final stage (methanogenesis). The threshold instability in some of these reactors relates to the unique nature of the various substrates, mixtures, and high concentration of biodegradable organic matter as well as the onset of hydrolysis and acidogenesis in the reactors [13]. However, the reactors functioned normally because the initial pH (Figure S1B) remained stable and within the optimal range of 6.5–8.2 for AD. This is most likely related to the sufficient presence of inoculum, which provided the required buffer to prevent further pH drop during acidogenesis in both mono- and co-digestion reactors.

Furthermore, initial TAN levels (Figure S1C) were also generally within acceptable ranges in AD. When present in high concentrations, ammonium can directly inhibit biogas production during AD [42]. Various studies have cited different inhibitory thresholds for TAN because it depends on factors such as substrate composition, temperature, pH, alkalinity, and acclimation period [42]. Final pH values were also within the prescribed range for methanogenesis. Overall, the performance of the reactors is deemed satisfactory judging by these parameters and indicators.

3.2.5. Kinetics Model Result and Comparison

Table 4 shows the parameters estimated using the first-order equation (FO), modified Gompertz model (GM), and logistic function (LF). While FO gave an R^2 value of 0.967–0.999, a range of 0.947–0.999 was obtained for MG, while the LF gave a range of 0.936–0.999. These ranges show the selected models can adequately simulate the kinetic patterns of biomethane production. However, to better compare the estimated values, the difference between the predicted value and the experimental value ($\Delta^c/_o$) was derived as seen in Table 4. The FO exhibited a higher upper value ($\Delta^c/_o$ = 2.0–24.7%), while MG ($\Delta^c/_o$ = 0.2–9.3%) and LF ($\Delta^c/_o$ = 0.6–9.8%) had less than 10% deviations, which shows the ability of MG and LF to provide a more accurate estimation than FO. Based on the results of this study, the ultimate methane production potential β_o (mL gVS^{-1}) was observed to be under-estimated more by LF (10 cases out of 12 cases) than the MG (8 cases out of 12 cases). Over-estimation occurred in LF in two cases compared to MG, which occurred in four cases.

The FO is mostly applied to estimate the hydrolysis constant, k (day^{-1}), of a substrate. Hydrolysis is considered the rate-limiting step in AD, and k is a measure of the biodegradability of substrates. A higher k value is more desirable because this usually indicates a higher degradation rate [32]. Considering the estimated parameters for k in the mono-digestion, TWAS (k = 0.16 day^{-1}) and OFMSW (k = 0.16 day^{-1}) are easily labile and, as expected, showed the fastest hydrolysis rate. On the other hand, SS, which had the highest methane yield, had the lowest k value of 0.07 day^{-1}. One reason for this could be because FOG from most WWTP is considered to be a slowly biodegradable particulate organic matter [43]. The k value of SS could mean that low hydrolysis can also promote high methane yield [11]. The estimation of the k value becomes a little challenging in the co-digestion mixtures due to the varying fractions of SS, OFMSW, and TWAS. Relative to TWAS alone, the k value reduced in all co-digestion mixtures, suggesting that hydrolysis became slower, perhaps due to the presence of multiple organic components, thus requiring more time for hydrolysis.

The lag phase shows how long it took for the microbial population to modify and take advantage of the new environment. Generally, the LF model estimated longer lag phases than the MG model. The λ values of zero meant methane production was started quickly in TWAS and OFMSW, perhaps due to quicker hydrolyzation of the organics, which facilitated onward bio-accessibility of VFA to methanogens for methane production. On the other hand, the lag experienced by SS (λ = 61.6 hrs (MG); λ = 71.7 hrs (LF)) may be related to known the slowly biodegradable particulate organic matter present in FOG waste [44].

Grosser [19] reported a longer lag phase during GTS digestion (λ = 168.2 hrs (MG) and λ = 198.5 hrs (LF)).

When it comes to the co-digestion mixtures, the LF model estimated longer lag phases than the MG model, thus making it challenging to draw a suitable conclusion. However, the unique composition of the mixtures might have also affected the lag time. In addition, it is reasonable to assume that, compared to the single substrates, the addition of co-substrates increased the lag time in the majority of the mixtures, even though the methane yields improved [8]. Within the mixture groups, there was a reduction in the lag phases. Within the 20%SS group, a decrease in the %OFSMW led to a reduction in the lag phase, whereas the opposite is true for the 40%SS group. Another factor in the 40%SS group is the higher concentration of SS, which may have contributed to the longer lag. Therefore, to avoid longer lag phases, the co-substrate of FOG wastes must be carefully selected. The Rm value (mL gVS^{-1} day^{-1}) indicates the rate at which methane and methanogenesis occur in the reactor. Fairly similar values were estimated by both MG and LF models. In Table 4, SS had the highest value of 43.44 mL gVS^{-1} day^{-1} (MG) and 43.68 mL gVS^{-1} day^{-1} (LF). Despite the long lag phase in this reactor, this result shows that methanogens consumed the organic acids in this reactor fairly quickly, thus leading to a faster methane production rate and a higher methane yield. Grosser (2018) reported lesser values of 28.2 (MG) and 31.67 (LF) for a GTS sample. The Rm values for OFMSW (25.92 mL gVS^{-1} day^{-1} (MG), 24 mL gVS^{-1} day^{-1} (LF)) were more than the rates reported in TWAS (18.24 mL gVS^{-1} day^{-1} (MG), 16.8 mL gVS^{-1} day^{-1} (LF)). For OFMSW, Grosser [19] reported higher values of Rm (MG = 34.16 mL gVS^{-1} day^{-1}, LF = 33.86 mL gVS^{-1} day^{-1}) than what was obtained in this study. The same study showed sewage sludge had higher values (MG = 24.88 mL gVS^{-1} day^{-1}, LF = 26.88 mL gVS^{-1} day^{-1}) than the TWAS Rm values estimated by the models. When it comes to the trinary mixtures, the Rm value improved highest in 40%SS + 50%TWAS + 10%OFMSW with 28.56 mL gVS^{-1} day^{-1} (MG) and 27.36 mL gVS^{-1} day^{-1} (LF), thus confirming the earlier position that the addition of SS and OFMSW to TWAS can help improve the methane production rate during co-digestion. However, the Rm values were not positively impacted when TWAS was added to all the trinary mixtures of 20%SS.

3.3. Microbial Analysis

The taxonomic profiles and composition of the microbiome are useful in providing valuable insight into the digester's performance [45]. In this study, comprehensive shotgun metagenomic sequencing was performed to understand the effect of the various co-digestion scenarios on the microbial ecology in both the initial and final phases of the process. Bacteria communities dominated 92.32% of the population, while 6.64% belonged to the archaea communities. Eukaryotes and viruses were 0.865% and 0.172%, respectively.

On the phylum level, *Euryarchaeota* was observed as the most abundant of the archaea community, whereas *Proteobacteria*, *Candidatus Cloacimonetes*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes* were the most abundant bacteria groups. To better understand the community structure, Figure 3 depicts the top phyla groups observed in this study.

Many of these microbial groups have been previously identified and associated with food waste, FOG, and sewage sludge degradation in mesophilic and full-scale studies [46–48].

Proteobacteria was the most dominant phylum at the initial phase in both monosubstrates (26–31.1% at the initial vs. 14.8–21.7% at the final) and co-digestion mixtures (29.7–30.3% at the initial vs. 20–21.7% at the final). Between the initial and final phases, the abundances reduced in both mono- and co-digestion modes, with the highest reduction of 50.1% observed in SS as a single substrate, which reduced from 29.7% to 14.8%. On the other hand, a 32.5% reduction in relative abundance was observed in 20%SS + 50%TWAS + 30%OFMSW when the relative abundance reduced from 29.7% to 20.1%.

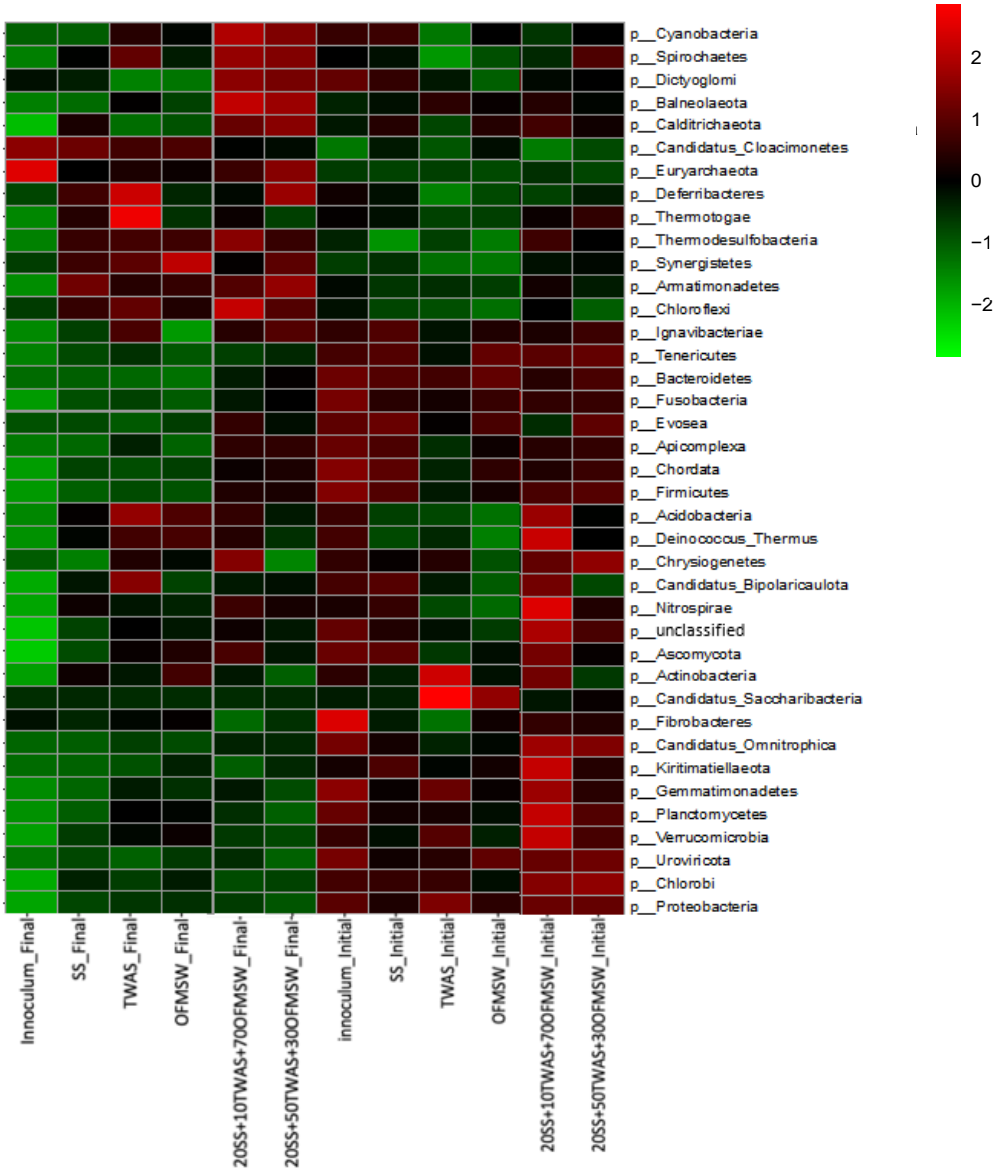

Figure 3. Heatmap of the top 40 phyla in both the initial and final phases of this study.

At the initial stage of single substrate digestion, the relative abundance of *Bacteroidetes* did not vary much and was lowest in TWAS, which had 10.6%, and highest in SS, with 11.8%. OFMSW was 11.2%. However, there was a reduction in the relative abundance at the final stage with 52.1%, 42.8%, and 48.8% in SS, TWAS, and OFMSW, respectively. Within the co-digestion mixtures studied, the relative abundances of *Bacteroidetes* were less in the final phases than in the initial phase, but they were of reduced magnitude when compared to the mono-substrates; 20%SS + 10%TWAS+70%OFMSW (9.8%) and 20%SS + 50%TWAS + 30%OFMSW (10.7%) were observed to have reduced by 19.6% and 18.6%, respectively.

Similar to *Bacteroidetes*, *Firmicutes* also assumed the same trend as that seen in both monosubstrates (7.4–9.6% at the initial phase vs. 5.5–6.7% in the final phase) and co-digestion modes (8.9–9.1% at the initial phase vs. 8.2–8.3 at the final phase).

The above results suggests that higher relative abundances of these organisms, especially at the initial stage, could mean that *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* are mostly the hydrolytic agents, and they partake in the hydrolysis of complex compounds to simpler and soluble monomers. *Proteobacteria* are also known to participate in the production of VFAs during acidogenesis [49].

Actinobacteria are mainly acidogens and usually promote the accumulation of VFA during AD. The trend of the relative abundance of *Cloacimonetes* shows there was an increase in the abundance between the initial and final phases. This phylum was the most dominant phylum in the final phase with 19.8–28.8% in the initial phase and 29–41.5% in the final phase of both monosubstrates and co-digestion mixtures. Within the mixtures, up to 29.7% was observed in the final phase of 20SS:10TWAS:70OFMSW. *Cloacimonetes* was mainly represented by the species *Candidatus Cloacimonas acidaminovorans*, which has been reported to be involved in syntrophic propionate metabolism and was detected to play an active role during AD of FOG containing wastes and sewage [50].

The analysis of the obtained sequence shows that in the archaea community, a majority (6.1%) of the taxonomic units were assigned to *Euryarchaeota*. *Euryarchaeota* is a deep branch mainly composed of methanogenic organisms [51]. Compared to the initial stage, the increase in relative abundance in the final stage could be an indication that methanogenesis was mainly occurring. Within the archaea community, the order *Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales* contribute to the final stage of methane production during AD [48].

Methanosarcinales comprises acetoclastic methanogens that cleave acetate to CH_4 and CO_2, while *Methanobacteriales* and *Methanomicrobiales* are more metabolically versatile and capable of utilizing hydrogen and several different carbon sources as electron donors [48,51]. Figure 4 shows the major methanogenetic genera of the archaea community observed in this study. These genera have been associated with methane production from food waste, sewage sludge, and FOG [51,52].

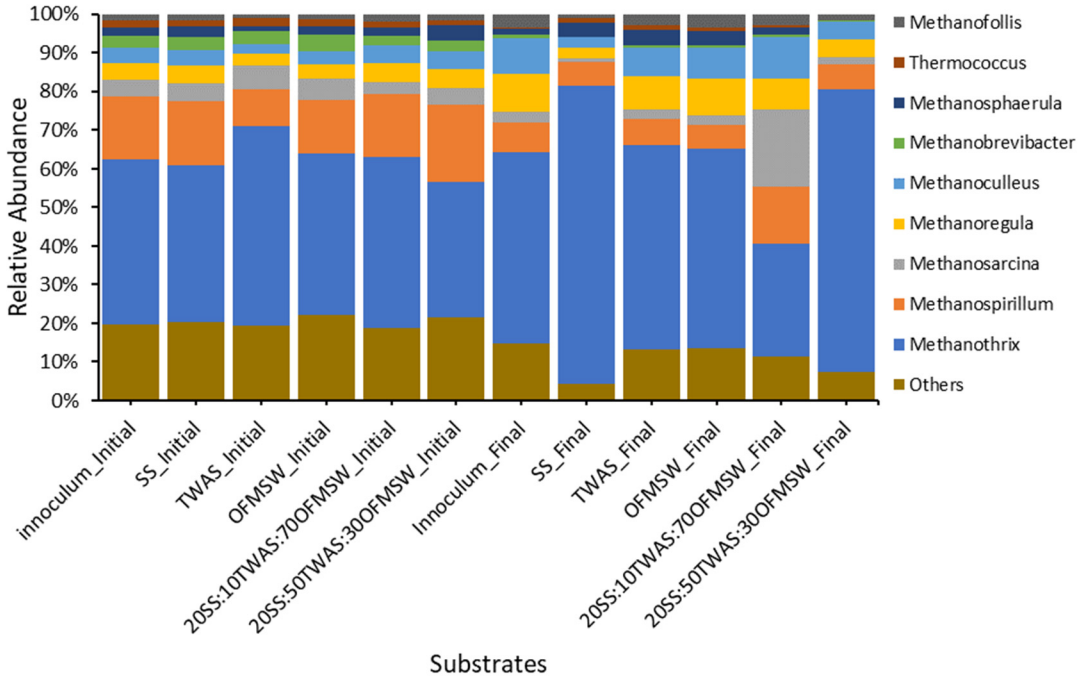

Figure 4. The relative abundances of the top 10 most prevalent genera in *Euryarchaeota* in both the initial and final phases of this study.

The genus *Mathanothrix* (synonymous with *Methanosaeta*) of *Methanosarcinales* was the most abundant in the majority of the reactor stages, followed by *Methanospirillum* (*Methanomicrobiales*) and then *Methanosarcina* (*Methanosarcinales*).

In the single substrates, *Methanosaeta* is observed to have dominated in both the initial and final phases. The highest relative abundance at the initial stage was observed in TWAS, with 52.3%. However, the final stage showed that *Methanosaeta* in SS accounted for as much as 79.8% which might have been a factor in the high methane production observed in the reactor. Within the mixtures investigated, the community shift was observed in the 20%SS

+ 10%TWAS + 70%OFMSW reactor, where the abundances of *Methanosaeta* (44.4%) and *Methanospirillum* (16.1%) at the initial stage reduced to 28.6% and 14.5%, respectively, in the final stage. At the same time, *Methanosarcina* increased from 3.2% initially to 19.3% at the final stage. In addition, *Methanosaeta* in 20%SS + 50%TWAS + 30% is seen to have doubled in relative abundance as it increased from 35.2% at the initial stage to 71.4% at the final stage. This led to a major reduction in the relative *Methanospirillum* (20.4% at the initial vs. 6.1% at the final).

The prevalence of *Methanosaeta* is a likely indicator that methane production in most reactors was mainly achieved through the acetoclastic pathway. Moreover, the abundance of genera *Methanospirillum* and *Mathanosarcina* are also indicators that the hydrogenotrophic pathway to methane recovery also occurred in the reactors. However, the role of the genus *Mathanosarcina* in this study requires further investigation because they are known to be versatile in acetoclastic, hydrogenotrophic, and methylotrophic pathways during methane production. Part of the reason why *Methanosarcina* was outcompeted by *Methanosaeta* is that the latter is easily accumulated under mesophilic conditions at low acetate concentration [51]. Moreover, the genus *Methanosaeta* has been reported to possess a higher affinity for acetate than *Methanosarcina* [52]. The dominance of *Methanosaeta* was also reported by Arelli et al. [52] during the digestion of food waste and sewage sludge. Hydrogenotrophic methanogens including *Methanospirillum* along with *Methanoregula*, *Methanoculleus*, *Methanosphaerula*, and *Methanofollis*, all of the order *Methanomicrobiales*, were also observed.

The shift in community abundance in the co-digestion mixtures could be related to the unique combination of the substrate mixture because methanogenic archaea adapt differently depending on the substrates and environmental conditions. Overall, the proportion of the various substrates is seen to have influenced the microbial community in both the initial and final stages of the sequenced samples.

4. Conclusions

This BMP study investigated the feasibility of the supplemental addition of OFMSW and SS during the digestion of TWAS as a source of renewable energy as well as a means to utilize digester capacity in the early design life of plants. The results show that, compared to TWAS alone, biomethane yield and energy production were enhanced through co-digestion of TWAS with SS and OFMSW due to the benefit of co-digestion, positive synergism, and improved biodegradability. A mixing ratio of 40SS + 10TWAS + 50OFMSW (VS basis) proved to be the best-performing mixture. The application of the kinetic models to the methane data indicated that the addition of OFMSW and SS to TWAS helped improve the methane production rate. In addition, the lag phase of SS was reduced by over 95% during co-digestion with TWAS and SS. Percentage VS removal did not lead to an increased methane production rate during the trinary co-digestion with TWAS. Sequencing results show that the microbial communities were dominated by *Proteobacteria and Cloacimonetes*. *Euryarchaeota*, comprising the archaea community, mainly dominated the acetoclastic methanogen of the genus *Methanosaeta*, followed by hydrogenotrophic methanogen *Methanospirillum* in both mono-digestion and co-digestion modes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9030237/s1: Figure S1: (A) Average initial and average final TOTAL VFA/TA ratios in all the reactors. (B) Average initial and average final pH values in all the reactors. (C) Average initial and final total ammonia nitrogen (TAN) concentration in reactors.

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